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Alginate esters via chemoselective carboxyl group modification

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ABSTRACT

Alginates are (1 → 4) linked linear copolysaccharides composed of β-D-mannuronic acid (M) and its C-5 epimer, α-L-guluronic acid (G). Several strategies for synthesis of carboxyl modified alginate derivatives exist in the literature. Most of these however employ aqueous chemistries, such as carbodiimide coupling reactions. Based on our recently discovered method for homogeneous dissolution of tetrabutylammonium (TBA)-alginate, we now describe use of tetrabutylammonium fluoride (TBAF)-based two component solvent systems as media for synthesis of carboxyl-modified alginate esters. Partially and fully esterified benzyl, butyl, ethyl, and methyl alginates were synthesized via reaction with the corresponding alkyl halides. The newly synthesized derivatives were soluble in polar aprotic solvents without the addition of TBAF. Saponification was performed to demonstrate that alkylation was completely regioselective for carboxylate groups in preference to hydroxyl groups to form esters. We demonstrate the utility of these alginate esters to enhance aqueous solubility of the flavonoid naringenin by formation of solid dispersions.

1. Introduction

Alginates are unbranched polysaccharides consisting of 1 → 4 linked β-D-mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G). They are isolated from one of two major sources – algae such as kelp, or as an exopolysaccharide of bacteria such as *Pseudomonas aeruginosa*. The alginate microstructure is unique and comprises of M-blocks and G-blocks interspersed with MG sequences (MG-blocks). Source and species dependent variability exists between alginates in terms of their copolymer composition, sequence and molecular weights. This diversity is often used as a tool to control final properties in a number of natural functions. For example, in *Laminaria hyperborea*, the stipe and holdfast require high strength and rigidity and therefore contain alginates having higher fractions of G residues. In contrast, the leaves of the plant which require flexibility for flotation in streaming water possess alginates with lower fractions of G residues. Due to the abundance of algae in water bodies, there is a large amount of alginate material present in nature. Industrial alginate production is approximately 30,000 metric tons annually, and is estimated to comprise less than 10% of the biosynthesized alginate material (Draget, 2009). There exists considerable additional potential to design sustainable biomaterials based on alginates.

One of the most obvious and effective ways to design high performance biomaterials is by chemically reacting the functional groups available on the alginate backbone. Alginate being a water soluble polysaccharide in salt form, the use of aqueous chemistries such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydrosuccinimde (EDC/NHS) coupling reactions to make esters/amides is a widely used strategy (Pawar & Edgar, 2012). While aqueous phase reactions can be a good way to modify polysaccharides, they do not match the versatility that organic phase reactions can offer. This is clearly demonstrated through the rich body of literature published on organic dissolution and derivatization of cellulose (Liebert, Heinze, & Edgar, 2010). The fact that the body of literature on alginate derivatization is much smaller is due in part to the presence of ionizable carboxylic acid functionalities on the alginate backbone. These charges play a crucial role in dictating alginate organic solubility; in fact, until recently no organic solvent systems had been described that would permit full dissolution of alginate. Addressing this key problem, we have recently shown that it is possible to dissolve alginates in two-component organic media consisting of polar aprotic solvents (such as DMSO, DMF, DMAc or DMI) and the salt tetrabutylammonium fluoride (TBAF) (Pawar & Edgar, 2011). Water soluble alginate acetates up to DS_{acetyl} ~1.0 were synthesized by reaction of alginate hydroxyl groups in these solvent systems.

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A disadvantage with the TBAF based two-component solvent systems is that the salt is hydrated and there is always a small percentage of water present in the mixture. Furthermore, it has been shown by our group that TBAF is useful as a reagent for regioselective deacylation of cellulose esters (Xu & Edgar, 2012). Although not yet experimentally confirmed, a similar deacylation reaction occurring in situ could be the source of the limitation we have observed in alginate acylations, where alginate acetate product DS_{acetyl} has not exceeded ~1.0. We would therefore like to develop alginate derivatives that would dissolve in organic media without the addition of TBAF. From our past studies, no single component organic solvent (including ionic liquids) is capable of fully dissolving tetrabutylammonium (TBA) salts of alginic acid. The ability to dissolve alginates in water-free organic media would be advantageous in two ways. Firstly, it would permit the use of organic reagents that are incompatible with water, thereby enabling new pathways to alginate functionalization. Secondly, it would enable solvent-based processing methods, which may extend the applicability of alginates beyond the current scope (which involves mostly aqueous processing).

Substitution of alginate at the carboxyl group to form an ester is one potential approach to the preparation of derivatives with such properties. We are aware of two prior instances where alginate esters have been synthesized via carboxyl group modification, neither of which involves complete dissolution of alginate in organic solvents (Pawar & Edgar, 2012). The first derivative is a commercially available alginate ester, propylene glycol alginate, synthesized by reaction with propylene oxide. The second set of reports has detailed the reaction of TBA-alginate with dodecyl bromide to prepare dodecyl alginate in DMSO (Babak, Skotnikova, Lukina, Pelletier, Hubert, & Dellacherie, 2000; Leonard, Rastello de Boisseson, Hubert, & Dellacherie, 2004; Pelletier, Hubert, Lapicque, Payan, & Dellacherie, 2000). However, the maximum $DS_{dodecyl}$ value reported was 0.12. Our hypothesis was that by dissolving TBA-alginate homogeneously in TBAF containing solvent systems, and by using more reactive halide reagents, complete substitution (DS_{ester} = 1.0) would be possible, and intermediate degrees of substitution might be achievable by control of stoichiometry. Herein, we describe the use of bimolecular substitution nucleophilic (S_N2) reactions to esterify the alginate carboxylate groups, in order to seek access to fully as well as partially substituted derivatives. The solubility properties of the derivatives were studied and peak assignments were performed using alginates enriched in M and G residues.

One reason for our interest in more organic-soluble alginate carboxylate esters was our hypothesis that these amphiphilic polysaccharide derivatives might be useful as matrix polymers for amorphous solid dispersion (ASD) of drugs. Being able to dissolve alginates in organic solvents without the need for adding TBAF is a key processing step for the formation of ASDs. Therefore we report as part of this study the exploration of some of the alginate derivatives synthesized as amorphous solid dispersion polymers for the bioactive, poorly soluble, and poorly soluble flavonoid, naringenin (Nar).

2. Experimental

A. Materials: Four alginates were used for the study. We represent each as 'Mxxx', where M stands for mannuronate and xxx denotes the % of M residues in the sample. For example, M063 signifies a sample containing 63% M and 37% G residues. M100 alginate was isolated from an epimerase-negative mutant of *Pseudomonas fluorescens* (Chitnis & Ohman, 1990). M063 alginate, an alginic acid sodium salt from brown algae (low viscosity, Na-alginate) was from Sigma. M060 alginate was from FMC BioPolymer. M000 was an alginate containing G-blocks with an average $DP_n \sim 20$.

Water used in the experiments was deionized. Reagent alcohol (histological grade), hexanes (HPLC grade), DMSO (HPLC grade), DMF (HPLC grade), 5 N standardized NaOH solution, 1 N standardized HCl solution, NaI, KCl, potassium phosphate monobasic [KH_2PO_4] and ethyl acetate were purchased from Fisher Scientific (Fair Lawn, NJ). Benzyl bromide (98%) [BnBr], iodoethane (98%) [EtI], 1-iodobutane (98%) [BuI], 1-methyl-2-pyrrolidone (99.5%, extra dry) [NMP] and tetrabutylammonium hydroxide (TBAOH; 40 wt%, 1.5 M solution in water) were obtained from Acros (Fair Lawn, NJ). Deuterium oxide (99.9 atom% D; D_2O) containing 0.75% 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt for NMR, (±)-naringenin, and iodomethane [MeI] were obtained from Sigma–Aldrich (St. Louis, MO). KBr used for FTIR analysis was purchased from International Crystal Labs (Garfield, NJ). DMSO-d_6 for NMR was acquired from Cambridge Isotope Laboratories, Inc. (Andover, MA).

B. Synthesis of TBA-alginate: For conversion of Na-alginate to its protonated form, a literature procedure (Babak, Skotnikova, Lukina, Pelletier, Hubert, & Dellacherie, 2000) was followed with modifications. HCl (0.6 N, 120 mL) was mixed with reagent alcohol (120 mL) in a beaker. Na-alginate (8 g) was added to the mixture and stirred overnight at 4 °C. The mixture was filtered, then the solid (alginic acid) was washed thoroughly, sequentially with alcohol and acetone. The solid product was dried overnight in vacuo at 60 °C. Fully dried alginic acid was then dispersed in 200 mL water. TBAOH was added dropwise with continuous stirring at room temperature until the polymer was dissolved and the pH was adjusted to 10.0. If the solution was too viscous, small amounts of water were added until a final viscosity was reached at which the solution could be easily stirred. The aqueous TBA-alginate solution was then premixed with reagent alcohol. The premix was precipitated in a mixture of ethyl acetate–hexane. The solvent ratios used were aqueous alginate acetate:ethanol:ethyl acetate:hexane = 1:3:12:3. The precipitate was then filtered under vacuum and dried in vacuo at 60 °C. The ^{1}H-NMR and FTIR spectra for TBA-alginate are shown in the Supplementary Material as Figs. S1 and S2 respectively.

^{1}H NMR (500 MHz, D_2O) δ 5.82–3.46 (m, alginate backbone), 3.19 (t, $NC\underline{H}_2CH_2CH_2CH_3$ of TBA), 1.67 (m, $NCH_2C\underline{H}_2CH_2CH_3$ of TBA), 1.40 (m, $NCH_2CH_2C\underline{H}_2CH_3$ of TBA), 0.97 (t, $NCH_2CH_2CH_2C\underline{H}_3$). FTIR (KBr pellet method): 3400 cm^{-1}, hydroxyl O—H stretching; 2850–2960 cm^{-1}, aliphatic C—H stretching, 1610 cm^{-1}, carboxylate C—O stretching. Elemental composition of TBA-alginate M060 measured by elemental analysis: C, 56.46%; H, 10.46%; N, 2.86%. This composition corresponds to roughly 80% TBA and 20% Na salt. Yield: 9.22 g; 67%.

C. Synthesis of benzyl alginate: TBAF (0.1 g, 0.32 mmol) was dissolved in DMF (10 mL) at room temperature. TBA-alginate (0.15 g) was then added and the mixture stirred until the polymer was dissolved. The temperature was then raised to 80 °C. Benzylation was carried out using either BnBr or benzyl iodide (BnI). In case of BnBr, the necessary equivalents of reagent were directly added to the alginate solution. For BnI, the reagent was synthesized starting from BnBr and then added to the alginate solution. Upon reagent addition, the solution was stirred at 80 °C for 30 min, after which it was added to ethyl acetate (~150 mL) to precipitate the product. The precipitate was recovered and dried in vacuo at 60 °C. Dry crude benzyl alginate was then dissolved in DMF (~1.5 mL), precipitated in ethyl acetate, filtered and dried in vacuo; this step was repeated one more time. The dry solid obtained thereafter was benzyl alginate. The synthesis of BnI reagent is described as follows.

Benzyl iodide: NaI (0.535 g, 3.6 mmol) was dissolved in 5 mL of acetone (dried over 3 Å molecular sieves) under N_2 at room temperature. To this solution BnBr (0.43 mL, 3.6 mmol) was added. Upon

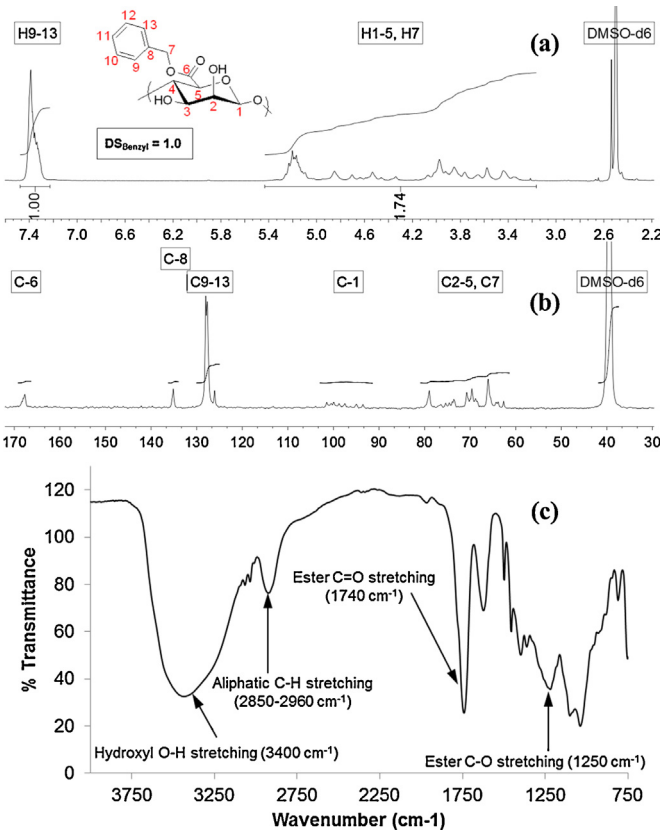


Fig. 1. (a) ^{1}H-NMR, (b) ^{13}C-NMR and (c) FTIR spectrum of benzyl alginate with DS_{benzyl} = 1.0. NMR solvent used was DMSO-d_6.

addition, NaBr precipitated as a white solid, which was filtered out and discarded. To the filtrate, 1 mL of DMF (solvent for benzyl alginate synthesis) was added, and acetone removed from the mixture using a rotary evaporator. BnI solution in DMF was thus obtained, and was immediately used for benzyl alginate synthesis.

For benzyl alginate shown in Fig. 1: ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.48–7.23 (m, H9-13), δ 5.43–3.16 (m, H1-5), 5.20 (m, H7). ^{13}C NMR (500 MHz, DMSO-d_6) δ 169.15–166.33 (m, C6), δ 135.24–125.33 (m, C8-13), δ 102.94–91.41 (m, C1), δ 80.88–61.39 (m, C2-5), δ 65.62 (m, C7). FTIR (KBr pellet method): 3400 cm^{-1}, hydroxyl O—H stretching; 2850–2960 cm^{-1}, aliphatic C—H stretching; 1740 cm^{-1}, ester C═O stretching; 1250, ester C—O stretching. Elemental composition: C, 46.62%; H, 4.70%; N, 0.04%; O, 41.99%. Yield: 90 mg; 94%.

Other esters such as methyl, ethyl and butyl alginate were synthesized using a similar procedure by replacing BnI with the respective alkyl iodides (such as iodomethane, iodoethane and 1-iodobutane). Reaction temperatures used for methylation, ethylation and butylation were 40 °C, 60 °C and 80 °C, respectively. ^{1}H, ^{13}C-NMR and FTIR spectra of these alginate derivatives are reported in the Supplementary Material as Figs. S15–S20.

D. Saponification of esters: Benzyl alginate with DS 1.0 (131 mg) was added to water (13 mL) forming a suspension. To this suspension, 1 M NaOH solution (1.3 mL) was added and stirred for 30 min. Over time, the alginate dissolved. It was then precipitated in reagent alcohol (~150 mL), filtered, the solid recovered and dried in vacuo at 60 °C. The dry solid was dissolved in water and re-precipitated in reagent alcohol, followed by filtration and drying in vacuo at 60 °C.

E. NMR analysis: For all alginate esters, the solid sample (40–50 mg) was dissolved in DMSO-d_6 (~0.7 mL) by applying heat. Spectra were acquired using a JEOL 500 MHz spectrometer with automatic shimming, and the acquisition temperature was 85 °C. Number of scans acquired was 16 for ^{1}H-NMR and DQF-COSY, 250 for HMQC and HSQC, and 13,000 for ^{13}C-NMR. All spectra were processed using MestReNova version: 8.0.2–11021. For water soluble samples, an automatic water suppression pulse was applied to suppress the residual HDO peak. For benzyl alginate, DS value calculation was carried out by integrating the benzyl aromatic peaks appearing at 7.48–7.23 ppm ($I_{Aromatic}$) against the region appearing around 5.43–3.16 ppm, which shows the backbone and benzyl CH_2 signals ($I_{Backbone+Methylene}$). $I_{Aromatic}$ accounts for 5(DS) protons while $I_{Backbone+Methylene}$ accounts for 7 + 2(DS) protons. The following formula was then used to calculate the DS value.

$$DS_{Benzyl} = \frac{7I_{Aromatic}}{5I_{Backbone+Methylene} - 2I_{Aromatic}} \quad (1)$$

To calculate the DS values for ethyl and butyl alginate derivatives, the methyl peak of the ester appearing at ~0.93 ppm (I_{Methyl}) was integrated against the region appearing at 5.95–3.44 ppm, which shows the backbone and ester CH_2 signals ($I_{Backbone+Methylene}$). I_{Methyl} accounts for 3× protons while $I_{Backbone+methylene}$ accounts for 7 + 2(×) protons. The following formula was then used to calculate the DS value.

$$DS_{Butyl/Ethyl} = \frac{7I_{Methyl}}{3I_{Backbone+Methylene} - 2I_{Methyl}} \quad (2)$$

The DS value of methyl alginate could not be calculated using ^{1}H-NMR due to the overlap of the methyl ester peak with the backbone region.

F. FTIR measurement: FTIR spectra were acquired using a Thermo Electron Nicolet 8700 instrument in transmission mode. Samples were prepared using the KBr pellet method. Alginate (1 mg) was mixed with 99 mg of KBr using a mortar and pestle. The mixture was compressed in the sample holder between two screws to form a KBr disk. 64 scans were obtained for each spectrum.

G. Elemental analysis: Measurements were performed using a %CHN analyzer with combustion/thermal conductivity detector (TCD) and a %O analyzer with pyrolysis.

H. DS measurement by titration: Titrations were performed by reacting alginate esters with excess NaOH to hydrolyze the ester groups. NaOH molecules remaining after hydrolysis were then back-titrated with HCl to measure the moles of ester present in the sample. For example in the case of benzyl alginate, 250 mg solid was weighed into a 50 mL 3-neck flask. To this, 25.0 mL of standardized 0.1 N NaOH solution was added and stirred overnight at room temperature. The water insoluble polymer dissolved over time. Potentiometric titrations were performed using a pH electrode (Inlab 413, Mettler-Toledo International, Inc.). The electrode was inserted into the 3-neck flask, 0.1 mL aliquots of standardized 0.1 N HCl solution added, the mixture allowed to stir for 30 s and the pH measured. Total volume of HCl added was 30 mL. Two plots were made using the recorded data. pH was first plotted against the volume of HCl added. Derivative d(pH) was plotted in a second graph against the volume of HCl added. The inflection point was recorded as the volume at which peak maximum was attained in the d(pH) plot.

Moles of ester groups present in the sample used for titration (250 mg) was calculated as follows:

$$\text{Moles of ester(M)} = \text{Initial moles of NaOH} - \text{Moles remaining after hydrolysis} \quad (3)$$

DS was then calculated as:

$$DS_{Titration} = \frac{M}{\text{No. of uronic acid residues in sample } (N)} \quad (4)$$

where,

$$N = \frac{0.25}{(266 \times DS_{Titration}) + (198 \times (1 - DS_{Titration}))} \quad (5)$$

Eq. (5) was plugged in to Eq. (4) to calculate $DS_{Titration}$.

I. Preparation of solid dispersions: Three solid dispersions were prepared starting from three different alginate ester derivatives – ethyl, butyl and benzyl. In each case, the alginate derivative (180 mg) was dissolved in 4 mL NMP by applying heat. Once the solution was cool, naringenin (60 mg) was added and dissolved at room temperature. The solution was then precipitated in ice-cold water (100 mL) and filtered to recover the wet solid, which was then placed in the freezer overnight before lyophilizing.

J. Alginate ester solubility in water: To test the solubility of each alginate ester in water, the solid (4 mg) was added to D_2O (1 mL), heat applied and the mixture stirred using a vortex mixer. The suspension was passed through a syringe filter to separate the solid, while the filtrate was added to a 5 mm NMR tube for acquisition.

K. Nar calibration curves: To plot calibration curves, Nar was dissolved in NMP at a concentration of 1 mg/mL. The NMP solution was then diluted to concentrations of 0.02, 0.015, 0.01 and 0.005 mg/mL using buffer solutions. To each dilute solution, an alginate ester derivative was added at a concentration of 0.21 mg/mL. UV–vis spectra were then obtained for each solution. Two buffer solutions were used in the study: pH 6.8 and 1.2; for each of the two buffers, separate calibration plots were made for ethyl, butyl and benzyl alginate derivatives. The absorbance wavelengths monitored were 316 nm in pH 6.8 buffer, and 291 nm in pH 1.2 buffer. The calibration plots are reported in the Supplementary Material as Figs. S22–S24.

L. Measurement of % drug loading: Drug loading was measured by dissolving the solid dispersion in NMP at a concentration of 1 mg/mL. The NMP solution was then diluted to a concentration of 0.02 mg/mL in pH 6.8 buffer, and a UV–vis spectrum obtained. The absorbance at 316 nm was recorded and the concentration of Nar in the solution was determined using the Nar calibration plot. Drug loading was then calculated as follows:

$$\text{Percent Drug loading} = \frac{\text{Nar concentration in } \frac{mg}{ml} \text{ as measured by UV}}{0.02} \times 100 \quad (6)$$

M. Nar release studies: Nar samples (pure or solid dispersion) were dispersed in 100 mL buffer (pH 6.8 or 1.2) in amber glass flasks at Nar concentrations of 0.07 mg/mL, and stirred at room temperature. Aliquots (1.5 mL) were withdrawn at appropriate time intervals and replaced with 1.5 mL of fresh dissolution medium after each sampling to maintain constant volume. UV–vis absorption of each aliquot was recorded after centrifugation at 14,000 rcf (relative centrifugal force) for 10 min.

N. XRD analysis: Measurements were performed using a Bruker D8 Discovery X-Ray diffractometer at 40 kV voltage and 25 mA. Scanned angle was set at $5 < 2\theta < 40^\circ$ and scan rate was 2°/min.

O. Thermogravimetric analysis (TGA): Measurements were performed using TA Instruments Q500 thermogravimetric analyzer in an inert atmosphere using high purity nitrogen (99.999%) with a flow rate of 40 mL min^{-1}. Thermal decomposition of 9–10 mg samples was studied at a heating rate of 10 °C/min from ambient temperature to 600 °C. Thermogravimetric curves were analyzed by Universal Analysis 2000 software (TA Instruments), Version 4.5A, Build 4.5.0.5.

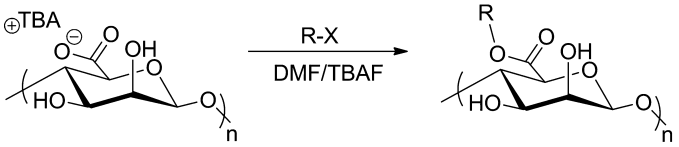
P. Differential scanning calorimetry (DSC): Measurements were performed using TA Instruments Q2000 differential scanning calorimeter equipped with a refrigerated cooling accessory. Samples (4–5 mg) were packed in non-hermetically crimped aluminum pans, and heated under dry nitrogen purge. For conventional DSC, samples were heated from 25 to 150 °C at 20 °C/min to eliminate moisture and relieve stress, then quickly cooled to 25 °C. Samples then were heated to 150 °C at 20 °C/min; this second heating scan was used to determine glass transitions. For modulated DSC, samples were heated to 170 °C at 5 °C/min, modulating ±0.5 °C every 40 s. Heating curves were analyzed using Universal Analysis 2000 software (TA Instruments), Version 4.5A, Build 4.5.0.5.

3. Results and discussion

As alginates are predominantly water-soluble polysaccharides in salt form, it has been a persistent effort in our laboratory to find diverse organic solvents capable of dissolving them. Our previous findings have shown that a minimum requirement necessary to solubilize alginates in organic media is the exchange of inorganic counterions (such as Na^+ or Ca^{+2}) with organic counterions (such as TBA^+). However, even after this requirement is satisfied, TBA-alginate can only dissolve in polar aprotic solvents (DMSO, DMF, DMI and DMAc) with the addition of the dissolution promoter TBAF. Nevertheless, these two-component solvent systems can serve as homogeneous reaction media, whereby it may now be feasible to react the available functional groups on the alginate backbone to synthesize derivatives with high degrees of substitution (DS). Our hope was that upon derivatization, the new derivatives would show improved solubility properties, and perhaps dissolve in single component organic solvents. Having single component solvents would not only eliminate the use of TBAF (and thereby avoid the almost unavoidable waters of TBAF hydration, as well as the potential for side reactions caused by the presence of fluoride ion) but also would permit easier processing for intended applications.

There are two available functional group types on the alginate backbone that may be used for derivatization. We have previously shown that upon acetylation of the —OH groups up to $DS_{acetyl} = 1.0$, the derivatives were water-soluble and showed no single component organic solubility. Furthermore, propionylation yielded alginate propionate up to $DS_{propionyl} = 0.31$, but its solubility was still limited to water. At this point, we hypothesized that while hydrophobic groups could be attached to the alginate backbone via —OH group modification, the charges imparted by the carboxylate moieties continued to provide significant hydrophilic character to the backbone, interfering with organic dissolution. The fact that we were unable to achieve the maximum theoretical DS of 2.0 upon acylation of alginate hydroxyls in these solvent systems was an additional hindrance to organic dissolution. We therefore adopted a strategy of carboxyl group esterification as an approach to synthesis of a new class of potentially organo-soluble alginate derivatives.

Selective reaction of charged carboxylate (COO^-) over neutral hydroxyl (—OH) groups is advantaged, since the former is a stronger nucleophile. The difference in nucleophilicity can be used to selectively react the carboxylate groups with substrates that can undergo S_N2 substitution. The ability to do so in our new alginate solvent systems (Pawar & Edgar, 2011) afforded the additional possibility of complete substitution on the carboxyl groups, as well as the possibility that no added base would be needed since alginate carboxyls are already ionized in our solvent systems. We therefore reacted alginate in solution with several halide reagents to prepare the corresponding esters. This reaction of carboxylates with halides is non-catalyzed and does not entail the use of extreme pH environments. Such conditions are favorable for preserving the molecular weight of the sensitive polysaccharide alginate during reaction. It is well known that alginates suffer a loss in molar mass in both strongly acidic (Smidsrød & Larsen, 1966; Timell, 1964) and strongly alkaline (Preiss & Ashwell, 1962;



Scheme 1. General reaction scheme for esterification of alginates using alkyl halides.

Tsujino & Saito, 1961; Whistler & BeMiller, 1960) solutions. Modification of alginates under mild pH conditions is therefore often preferred. A general reaction scheme for the esterification of alginate carboxylate groups using alkyl halides is shown in Scheme 1.

We first synthesized the benzyl ester by reaction of TBA-alginate with BnBr, achieving DS_{benzyl} = 0.44 upon performing the reaction at 80 °C for 30 min. The ^{1}H-NMR spectrum of the product is shown in Fig. S3 in the Supplementary Material. Knowing that the theoretical maximum DS_{benzyl} value is 1.0, this indicates that only 44% of the available carboxylate groups were substituted. What is more significant however is the fact that this is the first demonstration of the capability to react alginate with a hydrophobic organo-soluble reagent such as BnBr. Since BnBr is water immiscible, achieving the same level of substitution by performing the reaction in an aqueous medium could be anticipated as a challenge. This highlights the benefits of being able to dissolve alginates in organic solvents. In order to enhance the achievable DS_{benzyl}, we took the approach of replacing BnBr with a benzyl reagent that possesses a better leaving group. For example, when TBA-alginate was reacted with BnI at 80 °C for 30 min, we were able to synthesize benzyl alginate with DS_{benzyl} = 1.0. This to our knowledge is the first demonstration of complete substitution at the carboxyl group position to form alginate esters. Figs. 1(a) (^{1}H-NMR), (b) (^{13}C-NMR) and (c) (FTIR) show the spectra of benzyl alginate, DS = 1.0.

The ability to dissolve TBA-alginate in a variety of TBAF based two-component solvent systems has important implications for the preparation of alginate esters. This can be demonstrated through an example in which we first used DMSO/TBAF as reaction solvent for benzyl alginate synthesis. Our results indicate that while alginate carboxyl groups were benzylated, DMSO participated as a reactive solvent forming benzyldimethyloxosulfonium salts of alginate. A probable mechanism by which this reaction proceeds is shown in Fig. S4 of the Supplementary Material. The ^{1}H-NMR spectra of all benzyl alginates synthesized in DMSO/TBAF showed a consistent peak ~2.9 ppm, as indicated by (*) in Fig. S3 of the Supplementary Material. The reactivity of DMSO with alkyl halides is previously documented, wherein the alkyl halide can first react with the DMSO oxygen to give alkyloxydimethylsulfonium halide, which further rearranges to the more stable form of the salt yielding alkyldimethyloxosulfonium halide (Smith & Winstein, 1958). Due to the reactivity of DMSO with alkyl halides, DMF/TBAF proved to be a more suitable solvent system for alginate ester syntheses. Benzyl alginate with DS_{benzyl} = 1.0, free of side reactions could be prepared in DMF/TBAF by reaction of TBA-alginate with BnI at 80 °C for 30 min. As observed in Fig. 1(a), the peak around ~2.9 ppm was absent.

In order to prove that the reaction of benzyl iodide was indeed chemoselective toward alginate carboxylate groups, the product esters were hydrolyzed by treatment with aqueous NaOH. Any ether groups formed via —OH group reactions would not hydrolyze, being unreactive with aqueous alkali. We therefore compared the ^{1}H-NMR spectra of benzyl alginate before and after hydrolysis. Benzyl alginate is insoluble in the aqueous NaOH medium at first, but dissolves as it reacts. Therefore the NMR spectrum of benzyl alginate was acquired in DMSO-d_6, while that of the hydrolyzed product was acquired in D_2O (Fig. S5, Supplementary Material). It is evident that the aromatic peaks observed in the benzyl alginate spectrum completely disappear after hydrolysis. This confirms that the signals arising in the aromatic region for benzyl alginate arose only from carboxylate ester moieties, and not from benzyl ethers of the hydroxyl groups. This is strong support for the chemoselectivity of reaction of TBA alginate with alkyl halides at the carboxylate groups in preference to the hydroxyl groups.

Previous studies in our laboratory have shown that in the case of alginate acetates, ^{1}H-NMR integration methods lead to consistent overestimation of DS_{acetyl} values. This overestimation is a result of suppression of the residual HDO signal, which reduces the intensity of certain neighboring alginate backbone signals. A titration-based method was therefore developed to accurately measure DS_{acetyl}. While suppression of the residual HDO is not required when the NMR solvent is DMSO-d_6, it is still worthwhile to verify the accuracy of DS measurement by ^{1}H NMR. Benzyl alginate was therefore hydrolyzed in an aqueous NaOH solution of known molarity; the solid was initially insoluble in the medium but dissolved over time as the ester groups were hydrolyzed. The moles of NaOH remaining after hydrolysis were measured by titration against standardized HCl. The amount of titrant used was employed to calculate the moles of ester present, and therefore the DS_{benzyl}. Fig. S6 (Supplementary Material) shows the potentiometric titration and d(pH) plots for benzyl alginate. Both ^{1}H-NMR and titration methods yield the same value for DS_{benzyl} (DS 1.0), thus confirming that ^{1}H-NMR can be used as a reliable method for DS measurement for these alginate carboxyl esters.

Peak assignments for alginate esters can be performed with the aid of 2D-NMR spectroscopy. As a general observation, dissolving alginate derivatives in DMSO-d_6 for NMR resulted in better peak resolution compared to D_2O, where peak overlap appeared to be more severe. To facilitate interpretation of NMR spectra and assign signals arising from M and G residues, we took the approach of esterifying the homopolymers M100 and M000, and using their simplified spectra as guideposts for interpretation of copolymer spectra. The homopolymeric alginates are not commercially available, but M100 can be prepared from bacterial culture of epimerase-deficient bacteria (Chitnis & Ohman, 1990), while M000 can be obtained from extensive exposure of natural alginate copolymers to a series of alginate epimerases in vitro (Campa, Holtan, Nilsen, Bjerkan, Stokke, & Skjåk-Bræk, 2004; Hartmann, Duun, Markussen, Grasdalen, Valla, & Skjåk-Bræk, 2002a; Hartmann, Holm, Johansen, Skjåk-Bræk, & Stokke, 2002b; Høidal, Ertesvåg, Skjåk-Bræk, Stokke, & Valla, 1999). We then prepared butyl esters of alginates having varying M/G compositions in order to perform the necessary peak assignments. Figs. 2 and 3 show the stacked ^{1}H and ^{13}C NMR spectra of butyl alginates.

For peak assignment, we used a strategy wherein the anomeric signals were first identified based on their ^{13}C-NMR shifts. Then, using a combination of DQF-COSY (^{1}H–^{1}H coupling) and HMQC (^{1}H–^{13}C coupling) correlation spectroscopy techniques, we obtained information on the remaining peak assignments. We observed that for esters with DS values ranging between ~0.7 and 0.9, peak assignment for G residues was simpler than assignment of M residues. It is known that G residues show smaller sequential splitting and therefore lower resolution compared to M in ^{13}C-NMR spectra (Grasdalen, Larsen, & Smidsrød, 1977). This proved to be advantageous in the case of the butyl ester of M000 alginate, where only one anomeric peak was observed and was correlated to the corresponding esterified G backbone peaks. Conversely, for the butyl ester of M100 alginate, at least five separate anomeric signals were identified, and their correlations to corresponding M backbone peaks were challenging. Furthermore, cross peaks obtained in the 2D-NMR spectra were not well resolved in the case of M100. As the fraction of M residues in the backbone increased, the complexity of the NMR spectra increased correspondingly, and analysis became more challenging. We were nevertheless able to clearly

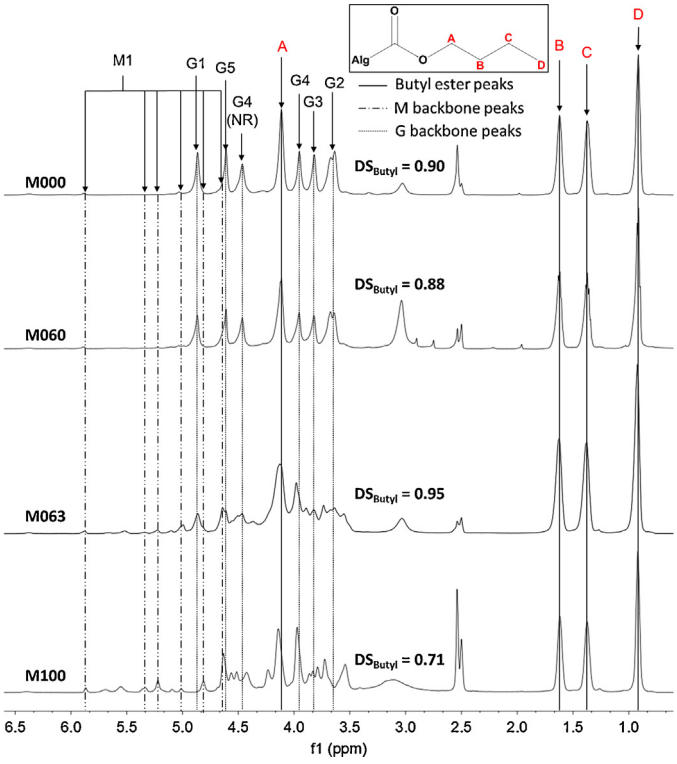


Fig. 2. Stacked ^{1}H-NMR spectra of butyl alginates of varying M contents. NMR solvent used was DMSO-d_6.

assign signals arising from G residues in all alginate samples. The DQF-COSY and HMQC spectra for all four alginates are shown in Figs. S7–S14 (Supplementary Material). In addition, all backbone peak shifts for G residues, and anomeric peak shifts for M residues are listed in Table S1 (Supplementary Material).

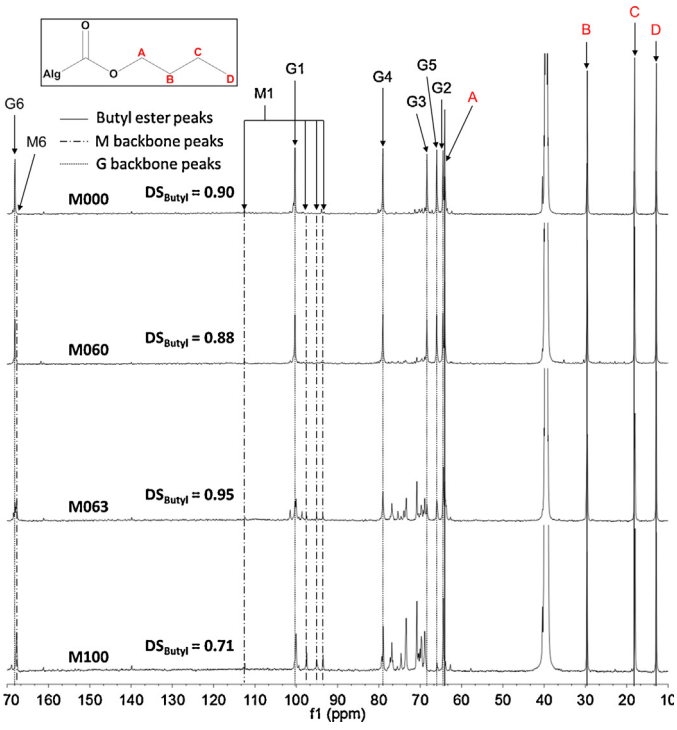


Fig. 3. Stacked ^{13}C-NMR spectra of butyl alginates of varying M contents. NMR solvent used was DMSO-d_6.

Synthesis of alginate esters using carboxylate modification can afford derivatives with a range of structures and properties based on the reagent and stoichiometry used. To demonstrate this, we synthesized methyl and ethyl alginate in addition to the benzyl and butyl ester derivatives. The ^{1}H and ^{13}C-NMR spectra of methyl, ethyl and butyl alginates are shown in Figs. S15–S20 (Supplementary Material). Table S2 shows their solubility properties vs. ester type and DS. All alginate esters with DS = 1.0 were soluble in polar aprotic solvents such as DMSO, DMF, NMP, DMAc and DMI; they were insoluble in methanol, ethanol, acetone and water. For partially esterified alginates with DS values ranging between 0.57 and 0.70, all samples showed complete solubility in polar aprotic solvents. Furthermore, they were all insoluble in water with the exception of ethyl alginate (DS = 0.62), which was partially soluble.

The utility of alginate esters can be demonstrated using an application that takes advantage of their ability to dissolve in organic solvents without the addition of a dissolution promoter such as TBAF. We were specifically interested in understanding whether these novel alginate derivatives had the potential to enhance the solubility of poorly water-soluble drugs such as Nar. Nar is the aglycone of naringin, a flavonoid that confers a bitter taste to citrus fruits. Among several potential Nar health benefits, it is well known to possess hypolipidemic and anti-diabetic properties, and is therefore of great interest in the treatment of high cholesterol and diabetes (Borradaile, de Dreu, Barrett, Behrsin, & Huff, 2003; Tsai, Huang, Mong, Kam, Huang, & Yin, 2012). In addition, like many other flavonoids, it is an antioxidant and is of importance in cancer prevention and treatment (Du, Jin, Han, Song, Zhang, & Liang, 2009). Recently, it was shown that Nar inhibits the secretion of hepatitis C virus (HCV) in infected patients, and is a regulator of cytochrome P450 enzymes, which are critical elements in drug metabolism (Goldwasser et al., 2011; Tsujimoto, Horie, Honda, Takara, & Nishiguchi, 2009). While Nar possesses favorable activity against a host of diseases, it suffers from poor water solubility, which is a cause of its low oral bioavailability. The Nar molecule is hydrophobic by nature (log P = 2.628), is highly crystalline with a melting point of 250 °C, and has low reported aqueous solubility (Tommasini, Raneri, Ficarra, Calabro, Stancanelli, & Ficarra, 2004). To be able to use Nar effectively in therapeutic formulations or dietary supplements, improving its aqueous solubility and bioavailability is critical.

One approach that has been of interest for enhancing the solubility of poorly water soluble drugs is through formation of ASD (Klein et al., 2007; Van Eerdenbrugh & Taylor, 2010). Solid dispersions have previously been defined as “a drug substance entrapped (or “dissolved”) in a carrier, usually a hydrophilic polymer”. By forming molecular dispersions of the drug with the polymer, drug crystal lattice formation is hindered, thereby increasing the extent of dissolution via supersaturation (Hancock & Parks, 2000; Miller, Beig, Carr, Spence, & Dahan, 2012). Important criteria for the selection of appropriate polymers for solid dispersions include their non-toxicity, miscibility with hydrophobic drugs, and a trigger mechanism for drug release. In addition, the polymer must be capable of providing stability against drug crystallization in both solid and solution states.

Polymer matrices chosen by previous ASD designers have been selected from polymers that had already been included in FDA-approved drug formulations. Polyvinylpyrrolidone (PVP) for example, is a synthetic polymer that is well suited for ASD formulations (Kanaze, Kokkalou, Niopas, Barmpalexis, Georgarakis, & Bikiaris, 2010). Another class of polymers that has shown promise is carboxyl-containing cellulose derivatives. These derivatives have high T_g values, availability of OH and COOH groups for secondary interactions with the drug, and the ability to tailor hydrophobicity using the type and DS of other substituents. Most well-known

among other cellulose derivatives are hydroxypropylmethylcellulose acetate succinate (HPMCAS) (Curatolo, Nightingale, & Herbig, 2009), carboxymethylcellulose acetate butyrate (CMCAB) (Posey-Dowty, Watterson, Wilson, Edgar, Shelton, & Lingerfelt, 2007; Shelton, Posey-Dowty, Lingerfelt, Kirk, Klein, & Edgar, 2009a), and more recently from our laboratory, cellulose acetate adipate propionate (CAAdP) (Ilevbare, Liu, Edgar, & Taylor, 2012a,b; Kar, Liu, & Edgar, 2011; Liu, Kar, & Edgar, 2012). While alginate is a carboxyl-containing polysaccharide and is recognized by the FDA to be safe as a direct food substance, it has not yet been explored for ASD formulations. In part this may be due to the difficulty in processing alginate formulations. Upon heating, alginate decomposes prior to melting, thus making thermal processing impossible. Furthermore, alginate insolubility in single-component organic media inhibits the use of formulation methods based on organic solvent solutions. Therefore we explored use of the carboxyl-modified alginate derivatives with enhanced solubility in single component polar aprotic solvents for ASD formation.

Several methods for the synthesis of ASDs are possible based on solvent processing. The first step involves dissolution of the drug and polymer in a common organic solvent or mixture of solvents. The solvent(s) can then be removed using methods such as evaporation under reduced pressure, for example by rotary evaporation, film casting, or spray drying. Each of these methods however requires the use of low-medium range boiling solvents such as acetone, methanol, methylene chloride, acetonitrile or ethanol. Unfortunately, the alginate esters we synthesized were not soluble in any of these solvents; their solubility was restricted to high-boiling polar aprotic solvents such as DMSO, DMF, DMI, DMAc and NMP. For this reason we chose a co-precipitation method, wherein the drug and polymer were dissolved in NMP, followed by co-precipitation in cold water. For the current Nar release study, we compared partially esterified benzyl, butyl and ethyl alginate derivatives listed in Table S2. Partially esterified alginates have free carboxylate groups on the backbone, which are essential to achieve pH dependent release of the drug from the matrix. In oral dosage formulation, the goal is frequently to prevent drug release in the low pH environment of the stomach, but promote release under the nearly neutral intestinal pH conditions. Free carboxylate groups are instrumental to the achievement of such targeted release. None of the three derivatives in question showed complete water solubility. However, we imagined that any partial water solubility might impact the percent drug loading of the resulting solid dispersion, and hence its release performance. The water solubility of each derivative was therefore tested using ^{1}H-NMR following exposure to D_2O. Fig. S21 (Supplementary Material) shows the spectra, wherein the alginate backbone region has been shaded gray. It is evident that the ethyl derivative shows partial solubility in D_2O, while the butyl and benzyl derivatives do not show any water solubility. Owing to this partial solubility of ethyl alginate in water, loss of polymer occurs during ASD preparation. A direct result of this is that actual drug loading is higher compared to the theoretically expected loading. In contrast, butyl and benzyl alginate solid dispersions yielded percent drug loadings that were lower than the theoretically expected values. Table S3 (Supplementary Material) shows the compositions of the SDs, and the theoretical and actual percent drug loading values.

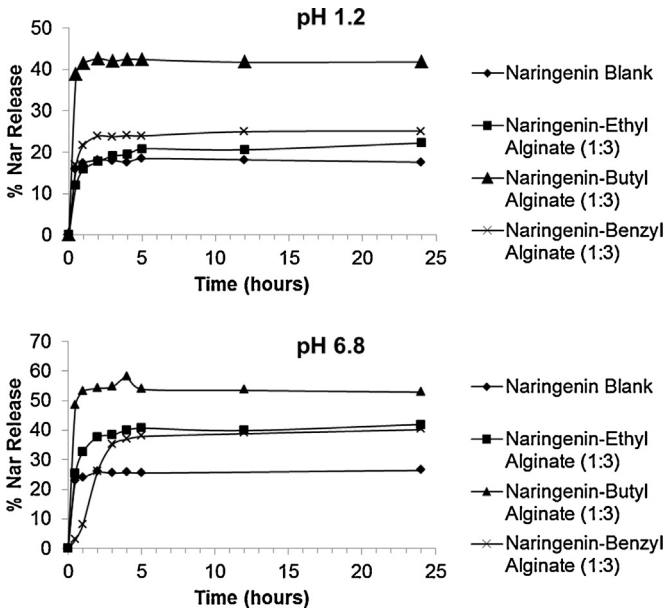


Fig. 4. Nar release profiles studied at pH 1.2 (top) and pH 6.8 (bottom).

We studied the release of Nar from the solid dispersions in aqueous buffer at pH 1.2 (simulating gastric environment) and pH 6.8 (simulating small intestinal environment). Fig. 4 shows the Nar release curves obtained. Compared to pure Nar (Blank), all three alginate derivatives showed enhancement in drug solubility at both pH values. Table 1 shows the maximum percent Nar release achieved for all SD formulations and the corresponding solubility enhancement factors. Ethyl and benzyl alginates showed solubility enhancement factors that were close to ~1.50, while that of butyl alginate ASD were slightly higher (ca. 2.30). We can compare these factors with those reported for carboxyl-containing cellulose derivatives (Li, Liu, Amin, Wegiel, Taylor, & Edgar, 2013). HPMCAS clearly yields the best improvement in Nar aqueous solubility with an enhancement factor of 14. CMCAB and CAAdP have factors of 5 and 4 respectively, and are closer to the values yielded by alginate esters.

In order for alginate derivatives to be competitive as matrix polymers, there is clearly a need for further improvement in their solubility enhancement factors. However, the purpose of this study is simply to demonstrate that modifying alginates in this fashion

Table 1
Maximum percent Nar released and solubility enhancement factors for the SDs studied.

SD	pH 1.2		pH 6.8	
	Maximum % Nar release achieved	Solubility enhancement factor	Maximum % Nar release achieved	Solubility enhancement factor
Nar Blank	15.84	–	23.84	–
Nar:Ethyl alginate (1:3)	22.19	1.40	41.22	1.73
Nar:Butyl alginate (1:3)	36.49	2.30	52.19	2.19
Nar:Benzyl alginate (1:3)	24.02	1.52	37.04	1.55
Nar/HPMCAS	–	–	–	14[a]
Nar/CMCAB	–	–	–	5[a]
Nar/CAAdP	–	–	–	4[a]

[a] Data reported by Edgar et al. (Li, Liu, Amin, Wegiel, Taylor & Edgar, 2013).

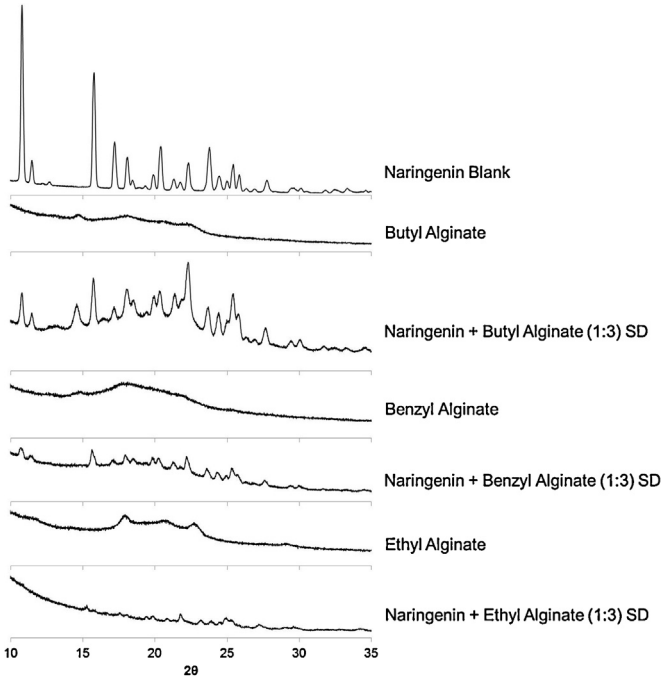


Fig. 5. XRD plots for crystalline Nar, alginate esters and their respective drug dispersions.

can lead to derivatives capable of solubility enhancement of poorly water-soluble drugs. Going forward, there may be several avenues possible for performance optimization. First, the ability to dissolve alginates in low boiling solvents can be expected to facilitate ASD formation by permitting the use of solvent evaporation techniques such as film casting, vacuum evaporation and spray drying. Solvent evaporation is perhaps the most flexible, practical technique for SD formation; melt extrusion is often preferred when it is practical, but it is restricted to cases where both drug and polymer have sufficient thermal stability. Extending the solubility profiles of alginate derivatives to permit dissolution in low-boiling solvents should be possible by functionalization of the available —OH groups in addition to the —COOH groups. Furthermore, varying the DS and type of both carboxyl and hydroxyl substituents should allow fine-tuning of the hydrophilicity-hydrophobicity balance to achieve optimal performance.

Determination of the glass transition temperatures (T_g) of the alginate derivatives is important, since high T_g polymers restrict the mobility of drugs in ASDs even at high humidity, ambient temperature, and drug content, thereby stabilizing against solid phase crystallization. For all three alginate ester derivatives (benzyl, butyl and ethyl) no glass transitions were observed using conventional DSC up to temperatures of 150 °C (Supplementary Material, Figs. S28–S30). Even employing a modulated DSC technique up to temperatures of 170 °C (Supplementary Material, Fig. S31), we were not able to observe any glass transition. TGA measurements showed that the onset of degradation (T_d) in the three derivatives occurs in the range of 180–190 °C (Supplementary Material, Figs. S25–S27). The fact that no T_g was observed suggests that the glass transitions occur at temperatures higher than T_d. Alternatively, it may be possible that the glass transitions occur below T_d, but are too broad for observation. Partial ester substitution along the backbone, coupled with the complex nature of the alginate backbone could be responsible for such a broadening effect.

The presence of residual drug crystallinity in the solid dispersions was studied using X-ray diffraction. XRD plots for the crystalline form of Nar, the three alginate derivatives and their respective solid dispersions are shown in Fig. 5. While the solid dispersion spectra are dominated by amorphous halos, peaks arising from the crystalline Nar can be observed in all three solid dispersions prepared using benzyl, butyl and ethyl alginate. This is clear evidence that residual drug crystallinity is present in these solid dispersions. By developing ways to eliminate this residual crystallinity, we believe that the performance of these alginate derivatives in solubility enhancement can be greatly improved. Alginates that are derivatized at both the carboxyl and hydroxyl positions may be promising candidates for such applications, and their development is part of an ongoing effort in our laboratory. This data demonstrates in principle that alginate esters are capable of enhancing solubility of poorly water-soluble drugs, but there is ample room for performance enhancement using multitude of strategies. We therefore believe that alginates are interesting candidates for achieving improved dissolution and bioavailability of poorly water-soluble drugs.

4. Conclusion

With a growing emphasis on the use of renewable resource materials and the potential to harvest algae in abundance, alginates have great promise as the source of polysaccharide derivatives for demanding applications. Herein we have demonstrated completely chemoselective modification of the alginate carboxyl group to provide a variety of esters, with DS controllable by stoichiometry, that broadens the palette of available alginate derivatives and provides a new route for their synthesis. This work builds on our previous finding that TBA salts of alginate are soluble in two component polar aprotic solvent systems such as DMSO/TBAF, and is enabled by the fact that the more nucleophilic carboxylates of these organosoluble alginate salts react directly and exclusively with alkyl halide reagents. The resulting carboxyl ester derivatives, including benzyl, butyl, ethyl and methyl alginates, are interesting because they have good solubility in polar aprotic solvents such as DMSO, DMF, DMAc, DMI and NMP without addition of the dissolution promoter TBAF, revealing promise for applications for which the natural alginate polymer would not be suited.

Reaction of the TBA salt of alginic acid in these solvent systems with alkyl iodides is successful due to the high reactivity of the alkyl iodides. Such a reaction is also attractive since it does not require added strong base; a significant challenge in derivatizing alginates is their propensity to degrade under acidic, alkaline and other conditions. Modification reactions like this one, that substantially change alginate physical properties under relatively mild pH conditions, are valuable avenues to new derivatives of well-preserved DP.

The improved solubility of these alginate carboxylate esters in organic media creates potential utility for the preparation of solid dispersion drug delivery systems. Partially esterified benzyl, butyl and ethyl alginates were useful for their retained pH sensitivity, and could be combined with Nar by co-precipitation from polar aprotic solvents. The resulting solid dispersions do modestly enhance the aqueous solution concentration of naringenin, roughly 1.5–2×. A possible cause of the small degree of enhancement was revealed by XRD analysis to be undesired residual drug crystallinity in the alginate carboxylate ester solid dispersions. More hydrophobic alginate derivatives that may more effectively retard crystal growth and/or nucleation could be accessible by modifying the OH groups of the more soluble alginate carboxylate esters. More broadly, this new method for chemoselective alginate carboxyl modification opens the door to design of regioselectively modified alginates with tailored structure and hydrophobic/hydrophilic balance, revealing fresh possibilities for utilization of this abundant natural polysaccharide.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.08.014.

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